Organic cation transporter 2 overexpression may confer an increased risk of gentamicin-induced nephrotoxicity

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Abstract: Nephrotoxicity is a relevant limitation of gentamicin, and obese patients have an increased risk for gentamicin-induced kidney injury. This damage is thought to depend on the accumulation of the drug in the renal cortex. Obese rats showed substantially higher levels of gentamicin in the kidney than did lean animals. This study characterized the role of organic cation transporters (OCTs) in gentamicin transport and elucidated their possible contribution in the increased renal accumulation of gentamicin in obesity. The mRNA and protein expression levels of the organic cation transporters Oct2 (Slc22a2) and Oct3 (Slc22a3) were increased in kidney samples from obese mice fed a high-fat diet. Similarly, OCT2 (2-fold) and OCT3 (3-fold) showed increased protein expression in the kidneys of obese patients compared with those of nonobese individuals. Using HEK293 cells overexpressing the different OCTs, human OCT2 was found to transport [(3)H]gentamicin with unique sigmoidal kinetics typical of homotropic positive cooperativity (autoactivation). In mouse primary proximal tubular cells, [(3)H]gentamicin uptake was reduced by approximately 40% when the cells were coincubated with the OCT2 substrate metformin. The basolateral localization of OCT2 suggests that gentamicin can enter proximal tubular cells from the blood side, probably as part of a slow tubular secretion process that may influence intracellular drug concentrations and exposure time. Increased expression of OCT2 may explain the higher accumulation of gentamicin, thereby conferring an increased risk of renal toxicity in obese patients.

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Organic Cation Transporter 2 (OCT2-SLC22A2) overexpression confers an increased risk of gentamicin-induced nephrotoxicity

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Abstract

Nephrotoxicity is a relevant limitation of gentamicin and obese patients have an increased risk for gentamicin-induced kidney injury. Drug-induced kidney injury is thought to depend on the accumulation of the drug in the cortex. Obese rats showed substantially higher levels of gentamicin in the kidney compared with lean animals. This study characterized the role of organic cation transporters (OCTs) in gentamicin transport and elucidated their contribution in the increased renal accumulation of gentamicin in obesity. The mRNA and protein expression levels of the organic cation transporters Oct2 (Slc22a2) and Oct3 (Slc22a3) were increased in kidney samples from obese mice fed a high-fat diet. Similarly OCT2 (~2-fold) and OCT3 (~3-fold) showed increased protein expression in the kidney of obese patients compared with non-obese individuals. Using HEK293 cells overexpressing the different OCTs, human OCT2 was found to transport [^{3}H]gentamicin with unique sigmoidal kinetics typical of homotropic positive cooperativity (autoactivation). In mouse primary proximal tubule cells, [^{3}H]gentamicin uptake was reduced by 40%, when co-incubated with the OCT2 substrate metformin. The basolateral localization of OCT2 suggests that gentamicin can enter proximal tubular cells from the blood side, probably as part of a slow tubular secretion process that may influence intracellular drug concentrations and exposure time. Increased expression of OCT2 may explain the higher accumulation of gentamicin, thereby conferring an increased risk of renal toxicity in obese patients.

Introduction

Gentamicin and aminoglycosides in general are very effective in treating a variety of Gram-negative bacterial infections but their use has been limited by nephrotoxicity and ototoxicity (1, 2). However, the development of multidrug resistant infections has led this class of antibiotics to be reconsidered in a number of clinical situations (3). Aminoglycosides have a higher incidence of nephrotoxicity in obesity (4). As obesity has become more prevalent, with currently more than 36% of the US population obese, the overall risk of aminoglycoside-induced nephrotoxicity may increase (5, 6). A better understanding of the underlying mechanisms of aminoglycoside-induced nephrotoxicity could help to design efficient protective strategies.

Animal studies have demonstrated that obese and lean rats had similar plasma gentamicin concentrations, yet accumulation of the drug in the kidney was greater in the obese group (7). The kidneys are key organs in the elimination of drugs. The processes involved in renal excretion include glomerular filtration, tubular reabsorption and tubular secretion and are governed by several protein-mediated transport systems (8). The disruption of tubular reabsorption or secretion due to altered expression and/or activity of these carriers can alter the disposition of many drugs (9, 10). Obesity is associated with several pathophysiological changes (11, 12). As regards renal function, obesity has been reported as an independent risk factor for chronic kidney disease, leading to glomerulosclerosis and renal insufficiency (13-16). Nonetheless, the effects of obesity on the expression of transporters involved in tubular reabsorption and secretion are poorly explored.

We previously analyzed the gene expression pattern of a variety of renal transporters in obese

mice (16). The expression of several solute carrier 22 (SLC22) family members was altered in the kidney of high-fat diet (HFD) induced obese mice compared with that of lean mice. The SLC22 family comprises organic cation transporters (OCTs), organic carnitine/cation transporters (OCTNs), and organic anion transporters (OATs) (17). Aminoglycosides are polycations and are likely to require protein-mediated transport to cross the cell membrane. Studies in knockout mice showed that megalin-mediated endocytosis from the apical side is the main mechanism for gentamicin uptake in the proximal tubule, whereas in rats the endocytic uptake played only a marginal role in gentamicin renal transport (18-21), suggesting that multiple transport systems can be involved in gentamicin renal accumulation with varying individual contributions.

Our data indicate that the expression of OCTs at the basolateral side of proximal tubular cells is increased in obesity and that OCT2 (SLC22A2) mediates the uptake and retention of [^{3}H]gentamicin in our in vitro model. The data show that organic cation transporters could contribute to the transport of gentamicin and explain the higher incidence of gentamicin-induced nephrotoxicity in obesity (22).

Materials and Methods

Reagents

Gentamicin [^{3}H(G)] sulfate ([^{3}H]gentamicin) was purchased from American Radiolabeled Chemicals (St. Louis, MO), 4-(4-(dimethylamino)-styryl)-N-methylpyridinium iodide (ASP^+)) by Molecular Probes-Life Technologies (Carlsbad, Ca). Non-labeled gentamicin sulfate and non-labeled metformin were provided by Sigma-Aldrich (St.Louis, MO). All cell culture

reagents were purchased from Gibco (Parsley, UK).

Animal procedures

Female C57/BJ mice aged 6 weeks were randomly assigned to a high-fat diet (HFD; D12331; Provimi Kliba, Switzerland) or a chow diet (D12329; Provimi Kliba, Switzerland). All mice were sacrificed under anesthesia 16 weeks after treatment and their kidneys were harvested. Half of the kidney from each animal was snap frozen in liquid nitrogen and stored at –80°C for RNA and protein extraction. The other half was fixed with formalin for histological examination.

Measurement of plasma samples

Plasma samples were obtained from the tail blood of fasted mice. Plasma cholesterol and triglyceride (TG) levels were measured by the Amplex Red cholesterol assay kit (A12216, Thermo Fisher Scientific, Carlsbad, CA) and the triglyceride assay kit (ETGA-200, EnzyChrom, Aachen, Germany), respectively.

Patients

Twenty-eight patients of Asian ethnicity with Minimal Change Disease (MCD) of the kidney, as diagnosed by ultrasonography-guided percutaneous biopsy, were included. The exclusion criteria were: (a) Body Mass Index (BMI) > 40kg/m^2; (b) history of type 2 diabetes mellitus; (c) renal, cardiovascular (heart failure, previous myocardial infarction), liver or thyroid diseases; (d) use of drugs known to affect the parameters under investigation. Participants had stable body weight (weight change < 5 kg during the last 6 months). Demographic data on both groups,

such as gender, age, weight, and plasma lipids are shown in Table 3. BMI was calculated according to the WHO. Appropriate BMI for Asian Populations and the Criteria of Weight and BMI for Chinese Adults, in which BMI<24 is normal and BMI>= 28 is defined as obese.

Immunostaining

Kidney sections were cut at 3 μm and immunostaining was performed on paraffin sections using a microwave-based antigen-retrieval technique. The antibodies used in this study included OCT2-SLC22A2 (Clone # 640438, R&D SYSTEMS, MN), OCT3-SLC22A3 (ab124826, abcam, Cambridge, UK). Sections were treated with the Envision+ DAB kit (Dako, Denmark) according to the manufacturer's instruction. Six mice were analyzed per group. The intensity of immunostainings for OCT2 and OCT3 on human biopsies was assessed according to protocols previously described (23). Briefly, digital images of random high-power fields were analyzed by an unbiased observer. The cross-sectional areas with positive staining were determined using Adobe Photoshop (version C3 for Windows). Six high-power fields were analyzed per biopsy.

Western blotting

Lysates (20 μg protein) from kidney tissue were separated by SDS-PAGE and blotted on polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). The membranes were blocked with TBS containing 0.1% Tween 20 and 3% BSA for 1 hour at room temperature and incubated overnight at 4°C with the respective antibodies. Subsequently, the blots were washed with TBS containing 0.1% Tween 20, treated with horseradish peroxidase conjugated

131 secondary antibodies at room temperature for 1 hour, and developed using the ECL Plus
132 detection system (Amersham Biosciences, Little Chalfont, UK). The antibodies used in this
133 study included OCT2-SLC22A2 (Clone # 640438, R&D SYSTEMS, MN), OCT3-SLC22A3
134 (ab124826, abcam, Cambridge, UK).
135
136 *Isolation of primary proximal tubular cells*
137 Primary proximal tubular cells were isolated from kidneys of female C57/BJ mice as described
138 previously (23). Briefly, kidney cortices were dissected, sliced, minced, and digested in 0.25%
139 trypsin solution (Life Technologies BRL, Grand Island, NY) in a shaking incubator at 37°C for 1
140 hour. Trypsin was neutralized with growth medium (DMEM and 10% FBS containing 100 U/ml
141 penicillin and 0.1 mg/ml streptomycin). The suspension was pipetted and was passed through
142 a 100 µm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). The samples were
143 centrifuged (72.4 x g for 5 minutes) to pellet the tubules, washed with 10 ml of medium,
144 centrifuged, and washed twice more. The final pellet, consisting mostly of renal tubules, was
145 resuspended in culture medium (REBM bullet kit, Clonetics, Basel, Switzerland), plated onto
146 culture dishes and incubated at 37°C in a humidified atmosphere of 5% CO_2 with medium
147 changes every 2 days until the cells were confluent.
148
149 *Isolation of kidney and cell RNA and quantification of transcript levels*
150 Total RNA was isolated using the Trizol procedure (Thermo Fisher Scientific, Carlsbad, CA)
151 and quantified at 260 nm. Two µg total RNA was reverse transcribed using oligo-dT priming
152 and Superscript II (Thermo Fisher Scientific, Carlsbad, CA). First-strand complementary DNA

153 was used as the template for real-time polymerase chain reaction analysis with the Taqman®
154 assay (Thermo Fisher Scientific, Carlsbad, CA). Transcript levels, determined in two
155 independent complementary DNA preparations, were calculated and expressed relative to
156 villin or β-actin as housekeeping genes.
157
158 *Cell lines*
159 Wild-type HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)
160 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin at
161 37°C in a humidified atmosphere of 5% CO_2. HEK293 cells stably transfected with the human
162 OCT1, OCT2 or OCT3, kindly provided by Dr. Hermann Koepsell, Würzburg, were
163 supplemented with Geneticin G-418 (600 μg/ml) (24).
164
165 *Transport Studies in Intact Cells*
166 Uptake of the radiolabeled or fluorescent compounds was measured using a protocol
167 designed for the quantification of uptake in cells (25). Cells were seeded in 35-mm dishes
168 coated with 0.1 mg/ml poly-D-lysine (Sigma-Aldrich, St. Louis, MO). To perform the transport
169 assay, the medium was aspirated and cells rinsed twice with pre-warmed transport buffer (136
170 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 11 mM D-glucose
171 and 10 mM Hepes/Tris, pH 7.4). Cells were equilibrated in transport buffer at 37°C, the buffer
172 was aspirated and transport buffer containing the radiolabeled or the fluorescent compound
173 was added. Uptake was stopped by quick aspiration of the incubation cocktail followed by
174 extensive washing with ice-cold transport buffer. Cells were digested for 45 min with 1 ml of 1%

(v/v) Triton X-100; 500 μl of the lysate was mixed with 4 ml of Scintillation Liquid (Ultima Gold, PerkinElmer, Switzerland) and assessed for intracellular radioactivity. Protein content was determined by the bicinchoninic acid protein assay on a 25 μl aliquot.

To measure intracellular ASP^+, the fluorescence (λ_{ex} = 485nm, λ_{em} = 590nm) of a 150 μl aliquot was measured on the Twinkle LB970 microplate fluorometer (Berthold Technologies, Germany). Protein content was determined by the bicinchoninic acid protein assay on a 25 μl aliquot. OCT independent uptake was determined in WT-HEK293 cells, and subtracted from total uptake to quantify OCT mediated uptake.

Statistical Analysis

Comparisons for the transport assays were performed with the two-tailed Student's paired t-test. Statistics for mRNA and immunostaining expression assays, mouse and human patients' parameters were based on the two-tailed Student's unpaired t-test. All analyses were performed using GraphPad Prism (version 5.0 for Windows, GraphPad Software).

Study approval

Animal experiments and protocols conformed to the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health) and the Swiss animal protection laws and were approved by the Cantonal Veterinary Office in Zurich, Switzerland (study number 2012058). The human study was conducted according to the Declaration of Helsinki guidelines regarding ethical principles for medical research involving human subjects. All patients provided written informed consent, and the study protocol was

197 approved by the Scientific Ethical Committee of Shandong University, Jinan, China, where
198 patients were based (license number SDU2015033).
199
200 **Results**
201 *Body weight and plasma lipid levels in chow fed and obese mice*
202 Table 1 shows body weight and plasma lipid levels in chow and HFD fed mice. The body
203 weight of obese mice was 30% higher than that of chow fed mice. The mean blood cholesterol
204 and triglyceride levels in obese mice were significantly higher than those measured in chow
205 mice, indicating that 16 weeks of HFD treatment induced obesity and hyperlipidemia.
206
207 *Effect of diet-induced obesity on organic cation transporter expression in the kidney*
208 Following on from previous microarray analyses of kidney RNA samples from chow and HFD
209 fed mice (16), expression of a group of membrane carriers, compatible with aminoglycoside
210 transport, was changed in obese mice (Table 2). Expression levels of Oct1, 2, 3, Octn1 and of
211 the multidrug and toxin extrusion protein 1 and 2 (Mate1 and Mate2) were validated by mRNA
212 quantification. HFD increased the expression of Oct2 and Oct3 at both the mRNA and protein
213 levels (Figure 1 A and B), which was in line with the microarray results. Because it was
214 reported to play a major role in gentamicin renal accumulation in mice, mRNA level of megalin
215 was assessed. It can be seen that megalin expression level in HFD and chow mice was
216 comparable, suggesting that megalin cannot account for the higher accumulation of
217 gentamicin in obese. Immunostaining for Oct3 showed that this transporter is localized at the
218 basolateral membrane of renal proximal tubules. The intensity of Oct3 staining was higher in

219 the obese mouse kidney than in the lean control group (Figure 1 C).

220

221 *Human OCT2 and OCT3 show increased expression in kidney biopsies of obese patients*

222 To investigate whether the enhanced expression of Oct2 and Oct3 in the kidney of obese mice
223 is also evident in obese human subjects, immunostaining for OCT2 and OCT3 was performed
224 in renal biopsy specimens from non-obese and obese human subjects who exhibited overt
225 proteinuria >1g/day and had a diagnosis of minimal change disease. Patient characteristics
226 are shown in Table 3. In line with the mouse data, OCT2 and OCT3 staining was more intense
227 in the proximal tubules of obese patients compared to non-obese individuals (representative
228 examples are shown in Figure 2 A-D) and quantification confirmed the increased expression of
229 OCT2 (~2-fold) and OCT3 (~3-fold) in obese patients (Figure 2 C and F, respectively).

230

231 *OCT2 transports [^{3}H]gentamicin*

232 To understand the role of organic cation transporters (OCTs) in gentamicin uptake, HEK293
233 cells transfected with OCT1, OCT2 or OCT3 were co-incubated for 2 min (linear range) with 1
234 μM of ASP^+ (known OCT substrate) in the presence or absence of non-labeled gentamicin at
235 an extracellular concentration of 10 mM (25, 26). Figure 3 A shows that non-labeled
236 gentamicin reduced OCT2 mediated influx of ASP^+ by approximately 50% (89.6 ± 17.5 vs 42.8
237 ± 10.6 pmol/mg of protein, P=0.02). OCT1- and OCT3-mediated transport was not affected by
238 gentamicin. Figure 3 B shows the OCT2-mediated ASP^+ influx at three different concentrations
239 of substrate as a function of extracellular non-labeled gentamicin at the indicated
240 concentrations (Dixon analysis). It can be seen that the lines intersect on the x-axis ($-K_i$),

241 indicating that the nature of the inhibition was non-competitive (K_i = 38.2 ± 4.4 mM) and
242 suggesting that gentamicin and ASP^+ did not bind to a common binding site. Transport of
243 [^{3}H]gentamicin mediated by OCT2 was measured at an extracellular concentration of 10 μM
244 (Fig. 4 C). After a 30 minute incubation, the intracellular level of [^{3}H]gentamicin in
245 OCT2-HEK293 cells was markedly higher than in wild-type cells (25.9 ± 1.1 vs 16.0 ± 1.1
246 pmol/mg of protein, P=0.002), indicating that OCT2 transported [^{3}H]gentamicin at
247 pharmacological concentrations.
248
249 *Kinetics of [^{3}H]gentamicin influx mediated by OCT2.*
250 Influx of [^{3}H]gentamicin as a function of concentration mediated by OCT2 was assessed over 4
251 min and indicated saturability. However, non-hyperbolic kinetics rather than Michaelis-Menten
252 kinetics were observed. The line was best-fit to a sigmoidal substrate-velocity curve indicating
253 allosteric kinetics (Fig. 5 A and B). The non-linearity of the transformed equations
254 (Hanes-Woolf and Eadie-Hofstee plots) further confirmed the allosteric interaction of
255 gentamicin with OCT2 (Fig. 5 C and D). The concave upward curvature of the Hanes-Woolf
256 plot of the data (Fig. 5 B) and a Hill coefficient computed to be 1.6±0.2 was consistent with a
257 homotropic positive cooperativity (Fig. 5 B).
258
259 *Role of OCT2 in [^{3}H]gentamicin uptake in primary proximal tubular cells (PTCs).*
260 To better understand the contribution of the endogenously expressed OCT2 on gentamicin
261 transport, primary proximal tubular cells (PTCs) were isolated from kidneys of C57/BJ mice.
262 The purity of the PTC preparation was assessed by measuring the mRNA expression levels of

263 villin, Oct2, and Mate1 in PTCs and glomerular fractions (not shown). Metformin, a known
264 OCT2 substrate (27, 28) was used to inhibit the OCT2-mediated transport of [^{3}H]gentamicin. In
265 figure 6 A it can be seen that metformin in excess (10 mM) could abolish completely the
266 OCT2-mediated transport of [^{3}H]gentamicin in OCT2-HEK293 cells. Therefore, to assess the
267 contribution of endogenous OCT2 in the uptake of [^{3}H]gentamicin, the uptake of
268 [^{3}H]gentamicin at an extracellular concentration of 10 μM was measured in presence or
269 absence of 10 mM metformin. In figure 6 B it can be seen that in presence of metformin the
270 uptake of [^{3}H]gentamicin was reduced by ~ 40% (P=0.04) suggesting a substantial
271 contribution of OCT2 in the uptake of [^{3}H]gentamicin in proximal tubular cells.
272
273 **Discussion**
274 Obese people have an increased susceptibility to aminoglycoside-induced nephrotoxicity but
275 the mechanisms underlying this higher risk are unknown. Animal studies have demonstrated
276 that obese and lean rats had similar plasma gentamicin concentrations, yet accumulation of
277 the drug in the kidney was ~ 30% greater in the obese group suggesting that renal gentamicin
278 uptake could be increased in the obese (7). Megalin-mediated endocytosis from the apical
279 side has been demonstrated to be the main gentamicin uptake system in proximal tubules of
280 knock-out mice (18, 21). The mechanism proposed involves an initial weak binding to acidic
281 phospholipids on the brush-border membrane of the proximal tubule followed by accumulation
282 of megalin in lysosomes and in the endoplasmic reticulum (21, 29-33).
283 The mRNA expression level of megalin did not change in the kidney of our mouse model,
284 conversely the expression of the main organic cation transporters OCT2 and OCT3 was

markedly increased in the kidney of HFD-induced obese mice and in kidney biopsies from obese patients. Hence, in consideration of the cationic nature of gentamicin, we hypothesized that OCTs could contribute to gentamicin renal uptake and that their increased expression might explain the augmented levels of gentamicin in the kidney of obese individuals, underscoring the role of OCTs in gentamicin uptake in proximal tubular cells (7, 34). It has been shown that megalin is not required for gentamicin accumulation in the HK-2 human renal proximal cell line and that co-incubation of gentamicin and mepiperphenidol, a prototype organic cation transporter inhibitor, could prevent gentamicin-induced toxicity in LLC-PK_1 cells (35, 36). Along with this, in rats, megalin plays only a marginal role in gentamicin uptake, and yet, rats seem to be more sensitive than mice to gentamicin-induced kidney damage (18, 21), suggesting that other transport systems, besides megalin, contribute to the accumulation of gentamicin in the renal cortex and, subsequently, to the kidney damage.

Among the different organic cation transporters expressed in the kidney, OCT2 was found to mediate the transport of [^{3}H]gentamicin. Because OCT2 is expressed exclusively at the basolateral membrane, it appears to be an uptake system for gentamicin into proximal tubular cells from the blood circulation (17). In previous studies in dogs and rats, when the renal tubular handling of gentamicin was measured, no tubular secretion could be observed (37, 38). However, the same authors did not exclude a slow tubular secretory flux due to temporary sequestration of drug in the proximal tubular epithelium after uptake across the basolateral membrane (37). Whether gentamicin is (i) sequestered or (ii) free but not able to exit from the luminal side (e.g. poor substrate for the multidrug extrusion transporter, MATE1) is not clear. In either case, our study shows that gentamicin is actively transported by OCT2 as underscored

307 by the nephrotropism and ototropism of aminoglycosides, the main sites of expression of
308 OCT2 in the human body (1, 2, 39, 40).
309 Male gender, among others, was found to be a significant independent risk factor for
310 aminoglycoside-associated nephrotoxicity in a study on 1489 patients prospectively monitored
311 with individualized pharmacokinetic monitoring (41). Similarly, male rats were more prone than
312 females to gentamicin-induced nephrotoxicity (42, 43). OCT2 expression is induced by
313 testosterone, thus in general more abundant in males than in females (44). Notably, castration
314 attenuated the gentamicin induced renal damage in male rats (45). The major role of OCT2 in
315 tubular secretion is further demonstrated by the observation that bile duct ligation in rats
316 induced the renal protein level of Oct2 and increased net tubular secretion even without any
317 significant changes in the glomerular filtration (46).
318 Kinetic data for the OCT2-mediated transport of gentamicin were well modeled empirically by
319 the allosteric sigmoidal equation demonstrating positive homotropic cooperativity (Hill
320 coefficient > 1) with increased binding affinity upon binding of a second gentamicin molecule.
321 The structural complexity of the OCT binding pocket is known and for several substrates
322 including choline, tetraethylammonium (TEA), and 1-methyl-4-phenylpyridinium (MPP), two
323 binding sites have been described, but no cooperativity was reported (47-49).
324 Substrate-dependent inhibitory ligand interaction is a common characteristic for OCT2 (49).
325 The search for inhibitors that can disrupt such allosteric binding may potentially reduce the
326 accumulation of gentamicin in the cortex avoiding nephrotoxicity in the clinical setting.
327 Recently, metformin, a well-known OCT2 substrate, was demonstrated to prevent
328 gentamicin-induced nephropathy in rats. Because at that time a role of OCT2 in gentamicin

transport was not considered, the effect of metformin on the renal accumulation of gentamicin was not explored (27, 28, 50). However, in light of the current findings it is possible that metformin could, to some extent, reduce the accumulation of gentamicin in the kidney, and buffer the renal damage.

The exact mechanisms underlying the altered pharmacokinetics and toxicokinetics of gentamicin in obese patients have not been characterized. Here, we provide evidence that OCT2 could be an important delivery route for gentamicin from blood into the kidney. The role of OCT2 in gentamicin-induced nephrotoxicity proposed in this work, and underscored by the different clinical safety profile of gentamicin in defined cohorts of patients (e.g. male vs female, obese vs non-obese), should promote careful experimental design and kinetic interpretation of future inhibition studies aimed at defining suitable compounds that can specifically disrupt the gentamicin-OCT2 complex and thus protect patients from renal damage (4, 41, 42).

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Contributions: Z.G. conducted experiments, acquired data, analyzed data and wrote the manuscript. M.V. conducted experiments, acquired data, analyzed data and wrote the

manuscript. C.H., E.K., T.L. and J.Z. conducted experiments and analyzed data. G.K.-U. designed research studies, acquired funding, analyzed data and wrote the manuscript.

Figure Legends

Figure 1. Effect of diet-induced obesity on organic cation transporter expression in kidney. (**A**) Quantification of mRNA levels of genes associated with cationic drug transport in mouse kidney. *n*=6 mice / group. Data are expressed as mean ± S.E.M., student's t test, $^{*}p<0.05$. (**B**) Western blot analysis of Oct2 and Oct3 protein levels in kidney samples of chow and HFD fed mice. (**C**) Representative images of immunostaining for Oct3 on kidney paraffin sections from the two groups.

Figure 2. OCT2 and OCT3 expression in renal biopsy specimens from non-obese and obese human subjects with minimal change kidney disease. Representative images of immunostaining for OCT2 (**A** and **B**) and OCT3 (**D** and **E**) in kidney specimens obtained from non-obese (body mass index < 21 kg/m^2, **A**, and **D**) and obese (body mass index > 25 kg/m^2, **B** and **E**) patients with overt proteinuria (>1.0 g/d). Quantitative analysis of the positive staining of OCT2 (**C**) and OCT3 (**F**) per high-power field. Data are expressed as mean ± S.E.M., student's t test, $^{*}p<0.05$.

Figure 3. Inhibition of the ASP$^+$ OCT-mediated influx by non-labeled gentamicin. (A) Influx of 1 μM ASP$^+$ was assessed in OCT1, OCT2 and OCT3 stably transfected HEK293 cells at pH 7.4 over 2 minutes in the absence (control) or presence of non-labeled gentamicin at an extracellular concentration of 10 mM. Uptake in WT-HEK293 cells was subtracted from that in transfected cells to define the OCT-specific transport. Data are expressed as percentage of the control**. (B)** ASP$^+$ influx at the indicated extracellular concentrations (1, 5 or 20 μM) as a function of increasing extracellular concentrations of non-labeled gentamicin. The line is derived by plotting 1/v against the concentration of gentamicin (inhibitor) (Dixon analysis). Data represent the mean ± S.E.M. from three independent experiments.

Figure 4. Time course of the net uptake of [^{3}H]gentamicin in OCT-transfected cells. Uptake of [^{3}H]gentamicin at an extracellular concentration of 10 μM in OCT1- **(A)**, OCT2- **(B)**, OCT3-HEK293 cells **(C)** was measured as a function of time and compared with that in wild-type cells. Data represent the mean ± S.E.M. from three independent experiments.

Figure 5. Kinetic analysis of [^{3}H]gentamicin influx. Initial uptake of [^{3}H]gentamicin was assessed over 4 min at pH 7.4 in OCT2-transfected HEK293 cells. Data were corrected for uptake in wild-type cells. [^{3}H]gentamicin influx as a function of substrate concentration. The data best-fit to the sigmoidal allosteric plot derived from the equation $Y=V_{max}*X^h/(K_{prime} + X^h)$ **(A)**. Enlargement of the first part of the kinetic analysis **(B)**. A Hanes-Woolf **(C)** and a Eadie-Hofstee plot **(D)** of the data. Results are the mean ± S.E.M. from five independent experiments.

Figure 6. Inhibitory effect of metformin on OCT2-mediated transport. Uptake of [^{3}H]gentamicin at an extracellular concentration of 10 μM in OCT2-HEK293 cells was measured as a function of time in the presence or absence of non–labeled metformin. The results were corrected for uptake in wild-type cells and a representative experiment is shown **(A)**. Uptake over 5 minutes of [^{3}H]gentamicin at an extracellular concentration of 10 μM in the presence or absence of 10 mM non–labeled metformin was measured in primary proximal tubular cells (PTCs) isolated from kidneys of C57/BJ mice. Data are expressed as the percentage of [^{3}H]gentamicin transport in the absence of non-labeled metformin and represent the mean ± S.E.M. from four independent experiments. $^*p<0.05$ **(B)**.

Table 1. Characteristics of mice in the chow and HFD groups after treatment

groups	Chow (n=6)	HFD (n=6)
Plasma Cho (mg/dl)	68.43 ± 1.918	85.58 ± 2.343*
Plasma TG (mg/dl)	51.03 ± 3.703	62.23 ± 3.883*
Body weight (g)	19.50 ± 0.500	25.50 ± 1.225*

Abbreviations: HFD, high fat diet; TG, triglyceride; Cho, cholesterol. n=6 mice/group, data are expressed as the mean ± SEM, student's t test, $^{*}p<0.05$.

Table 2. Selected differentially expressed genes in kidney tissue of HFD mice versus chow mice

Gene Symbol	Gene Name	log2 Ratio	*p* Value
Slc22a1	solute carrier family 22 member 1 (Oct1)	0.07952	0.694
Slc22a2	solute carrier family 22 member 2 (Oct2)	0.2504	0.05479
Slc22a3	solute carrier family 22 member 3 (Oct3)	1.098	2.73E-05
Slc47a1	solute carrier family 47 member 1 (Mate1)	0.4208	0.0727
Slc47a2	solute carrier family 47 member 2 (Mate2)	-1.162	0.003459

Gene expression levels are reported as Log_2 of fold changes.

Table 3. Demographic information of patients with minimal change kidney disease

groups	BMI<24 (n=17)	28≤BMI<40 (n=18)
Gender (male/female)	10/7	10/8
Age (year)	50.80 ± 7.61	52.61 ± 3.33
Plasma Cho (mM)	3.822 ± 0.176	4.514± 0.177*
Plasma TG (mM)	0.865± 0.056	2.486± 0.501*
BMI (Kg/m^2)	23.42± 0.278	28.17± 0.421*

Abbreviations: BMI, body mass index; TG, triglyceride; Cho, cholesterol. Values represent mean ± S.E.M., student's t test, *p<0.05.

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